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13. ABSTRACT (Maximum 200 words) The gene (vgb) for the bacterial hemoglobin from Vitreoscilla has been cloned in Burkholderia strain DNT which can degrade 2,4-dinitrotoluene (DNT). The new strain, YV1, expressed vgb, grew better, survived longer, and degraded DNT faster than strain DNT. The addition of surfactants and succinate or yeast extract also stimulated DNT degradation up to 99.5%. Measurements of the oxygen kinetics for DNT degradation showed that for all strains the kinetics of oxygen uptake were biphasic but could be resolved into two components. Purified Vhb added to cell-free extracts of strain DNT stimulated DNT degradation, but not control bovine Hb. Regarding biodegradation of DNT in a soil slurry spiked with DNT, YV1 showed a slightly higher degradation rate (ranging from 5 to 18%). When vgb was cloned into E.coli containing the gene for DNT dioxygenase the resultant strain exhibited enhanced growth, viability, and degraded twice as much DNT under low aeration conditions. DNT dioxygenase has been partially purified by gel chromatography; it requires NADH, is stimulated by phospholipids, and inhibited at high ionic strength. Probing the oxygen binding site of Vhb using site-directed mutagenesis showed that the distal residue GlnE7 was not involved in stabilizing the bound oxygen.					
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Enclosed please find two draft versions, as per instructions in the "Administration of U.S. Air Force Grants and Cooperative Agreements for Basic Research," of our Final Technical Report for the period 01 May 95 to 31 March 98 for our AFOSR grant, "Bacterial Strain Improvement for Bioremediation," number F49620-95-1-0325. Two papers are also available if you want them, one published, the other submitted. We were not certain whether they should be included with this report, nor was it clear whether we should submit detailed data, including figures, tables, etc. We will be happy to supply these or any other information upon request.

Sincerely yours,



Dale A. Webster



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Research Title: BACTERIAL STRAIN IMPROVEMENT FOR BIOREMEDIATION

Grant Number: F49620-95-1-0325

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SUMMARY

The gene (*vgb*) for the bacterial hemoglobin from *Vitreoscilla* has been cloned in *Burkholderia* strain DNT which can degrade 2,4-dinitrotoluene (DNT). The new strain, YV1, expressed *vgb*, grew better, survived longer, and degraded DNT faster than strain DNT. The addition of surfactants and succinate or yeast extract also stimulated DNT degradation up to 99.5%. Measurements of the oxygen kinetics for DNT degradation showed that for all strains the kinetics of oxygen uptake were biphasic but could be resolved into two components. Purified VHb added to cell-free extracts of strain DNT stimulated DNT degradation, but not control bovine Hb. Regarding biodegradation of DNT in a soil slurry spiked with DNT, YV1 showed a slightly higher degradation rate (ranging from 5 to 18%). When *vgb* was cloned into *E. coli* containing the gene for DNT dioxygenase the resultant strain exhibited enhanced growth, viability, and degraded twice as much DNT under low aeration conditions. DNT dioxygenase has been partially purified by gel chromatography; it requires NADH, is stimulated by phospholipids, and inhibited at high ionic strength. Probing the oxygen binding site of VHb using site-directed mutagenesis showed that the distal residue GlnE7 was not involved in stabilizing the bound oxygen.

OBJECTIVES

The expression of the bacterial hemoglobin from *Vitreoscilla* in heterologous hosts generally improves growth, increases protein synthesis, and increases genetically engineered product formation under oxygen-limited conditions. The gene (*vgb*) for *Vitreoscilla* hemoglobin (VHb) was previously cloned and expressed in a benzoate-degrading strain of *Pseudomonas/Xanthomonas*. Using this model system it was found that the presence of VHb in the cells did indeed enhance the degradation of benzoate. Thus, the major objective of this grant was to extend this technology to bacteria that are capable of degrading toxic aromatic nitro compounds, specifically 2,4-dinitrotoluene (DNT). This would include cloning *vgb* into a strain of *Pseudomonas* that has been demonstrated to degrade DNT, cloning it into strains of *E. coli* which have been genetically engineered to contain individual genes of the DNT degradative pathway, and testing the resultant transformants for their ability to degrade DNT under various conditions, especially under oxygen-limited conditions. These objectives have been pursued and essentially all accomplished. In addition to the original objectives, experiments have been initiated which are designed to expand the findings from basic laboratory research to more practical field tests. This includes, for example, testing the genetically engineered bacteria that were produced for their relative abilities to degrade DNT in soil and water samples contaminated with this toxic waste material. Basic research into the mechanism by which *Vitreoscilla* hemoglobin (VHb) exerts its beneficial effects in these bacteria was also initiated during the tenure of this grant. This has included doing site specific mutagenesis experiments on the oxygen binding site of *Vitreoscilla* hemoglobin to study how changes in the structure of this pocket affect its function. Purification of the first enzyme in the DNT degradation pathway, DNT dioxygenase, has been initiated so that oxygen transfer from VHb to this enzyme can be studied as a model system.

STATUS OF EFFORT: ACCOMPLISHMENTS/NEW FINDINGS

The gene (*vgb*) for VHb was successfully and stably cloned into *Burkholderia* (formerly *Pseudomonas*) sp. strain DNT, which is able to degrade and metabolize DNT. This new strain, YV1, expressed *vgb*, grew better, and survived longer than the parent strain. This was true in both a rich medium (tryptic soy broth, TSB) and in a minimal salts medium containing 200 ppm yeast extract and 100 ppm DNT. In the latter medium, YV1 degraded DNT faster than the untransformed strain. The addition of surfactants stimulated the degradation of DNT by both strains. In minimal medium, additional carbon source, succinate or a larger amount of yeast extract, also led to increased DNT degradation.

Previous studies of DNT degradation by *Burkholderia* in Spain's laboratory have shown that there are three oxygenases at the beginning of the degradative pathway. Since it was expected that VHb may exert its stimulatory effect by increasing oxygen supply to one or more of these oxygenases (as well as to the terminal oxidases) measurements of the oxygen kinetics for DNT degradation were undertaken. The initial experiments used intact cells of both strain DNT and YV1. The results from both were similar: the kinetics of oxygen uptake were biphasic (and possibly higher order) but could be resolved into two components, each with a different K_m and V_{max} . At this time it is not possible to assign these values to specific enzymes, but experiments with *E. coli* strains, each containing the cloned gene for one of the oxygenases, should help to further resolve these results. Additionally, cyanide (at around 3 mM) appears to inhibit oxygen uptake by the respiratory chain terminal oxidases and DNT oxygenases differentially and may be useful in distinguishing the effect of VHb

on each. Experiments using cell-free extracts gave very similar results for the oxygen kinetics. An important finding was that the degradation of DNT by cell-free extracts of strain DNT was stimulated by adding purified VHb but not by control bovine Hb.

In batch reactor experiments using DNT at 200 ppm, neither strain was able to grow when DNT was the only carbon source. However, when yeast extract was added, both strains grew and degraded DNT; the optimum extra carbon dosage to degrade 1 mg of DNT was about 3.2 mg yeast extract. Under these conditions the degradation of the DNT was 99.5%. YV1 showed a slightly higher DNT degradation rate than strain DNT only at the initial stages of the experiments. In soil slurry experiments, soil was synthetically contaminated by spiking with DNT solution and biodegradation of DNT by w.t. strain DNT and YV1 was examined. The DNT degradation by both strains was 45 to 75% after seven days in the soil slurry. YV1 showed a slightly higher degradation rate (ranging from 5 to 18%) throughout the experiment.

The gene (*dntA*) for the first enzyme (DNT dioxygenase) in the DNT degradative pathway had been previously cloned in *E. coli* JS39. In this case, instead of DNT being mineralized the product of this enzyme reaction is 4-methyl-5-nitrocatechol (MNC) which can be detected by its yellow color. When *vgb* was cloned into strain JS39, the resultant strain, PF6, exhibited enhanced growth and viability at low oxygen in minimal medium containing DNT compared with the parent strain. It also converted twice as much DNT to MNC as strain JS39, but only under conditions of low aeration. Comparative kinetic studies were undertaken to see how the presence of VHb might affect DNT dioxygenase activity in vivo. With PF6, the V_{max} for DNT was three to four times that for JS39, indicating that the former strain contains three to four times as much enzyme as the latter. The V_{max} for oxygen was the same in both strains. The K_m 's for both DNT and oxygen were increased up to two times in PF6 compared to JS39; one interpretation is that VHb may actually be inhibiting the dioxygenase because it has a higher affinity for oxygen than the enzyme. This can be tested once the purification of DNT dioxygenase has been achieved and its affinity for oxygen measured.

The purification of DNT dioxygenase has been complicated by the sensitivity of the enzyme to ionic strength: it is inhibited even at ionic strengths as low as about 0.05 M, which limits the purification techniques that can be used. However, some purification has been achieved by gel chromatography. The enzyme requires NADH and its activity is stimulated by phospholipids. Other than its sensitivity to high ionic strength, it is fairly stable. Its purification will allow us to examine whether oxygen can be transferred from VHb to the dioxygenase and how this affects its activity.

In other research, the role of the amino acid residue, glnE7, in the function of VHb was probed using site-directed mutagenesis. It was believed previously that this residue stabilized the oxygen bound to the heme iron with a hydrogen bond. However, recent x-ray crystallographic studies indicated that this residue was not in the oxygen binding pocket as expected. The results from our laboratory confirmed this finding since replacing the gln with leu, which is incapable of forming a hydrogen bond, had little effect on oxygen binding of VHb. We are now in the process of examining tyrB10 which is also in the oxygen binding heme pocket.

PERSONNEL SUPPORTED:

Postdoctoral Research Associates: Dr. Sangeeta Patel

Graduate Students: Jui-Ming Lin

Hong Ma

Kwang-Woo Hwang

Jin Chung

Kyung-Jin Kim

Aysel Golbahar

PUBLICATIONS

K.L. Dikshit, Y. Orii, N. Navani, S. Patel, H.-Y. Huang, B.C. Stark, and D.A. Webster (1998) Site-directed mutagenesis of bacterial hemoglobin: the role of glutamine (E7) in oxygen binding in the distal heme pocket. *Arch. Biochem. Biophys.* **348**, 161-166.

S.M. Patel, B.C. Stark, K.-W. Hwang, K.L. Dikshit, and D.A. Webster (1998) Cloning and expression of *Vitreoscilla* hemoglobin in *Burkholderia* sp. strain DNT for enhancement of 2,4-dinitrotoluene degradation. Submitted to *Applied Microbiology and Biotechnology*.

Other manuscripts in preparation:

P. Fish, D.A. Webster, and B.C. Stark (1998) Effects of *Vitreoscilla* hemoglobin on the conversion of DNT to MNC in recombinant *E. coli* bearing the *dntA* gene.

S. Patel, B. C. Stark, and D.A. Webster (1998) Degradation of 2,4-dinitrotoluene is enhanced by the presence of the bacterial hemoglobin gene, succinate, and surfactants.

J.-M. Lin, P. Fish, B.C. Stark, and D.A. Webster (1998) Effect of *Vitreoscilla* hemoglobin on kinetic parameters for the biodegradation of 2,4-dinitrotoluene.

K.L. Dikshit, Y. Orii, H.-Y. Huang, B.C. Stark, and D.A. Webster (1999) Site-directed mutagenesis of bacterial hemoglobin: the role of tyrosine (B10) in oxygen binding and hemoglobin function.

J. So, B.C. Stark, D.A. Webster and K. Pagilla (1999) Biodegradation of DNT by *Burkholderia* in batch reactor and soil slurry.

K. Al-Khleifat, J.-M. Lin, F. Najmabadi, S. Patel, B.C. Stark, and D.A. Webster (2000) Purification and some properties of 2,4-dinitrotoluene dioxygenase.

INTERACTIONS/TRANSITIONS

In collaborative work with Professor K. Pagilla and graduate student Juho So in the Department of Chemical and Environmental Engineering, we are testing the genetically engineered bacteria that we have produced for their abilities to remove DNT from soil and water samples contaminated with this toxic waste material. These pilot experiments are expected to be followed by actual field testing. We

also have an on-going collaboration with K. Dikshit (Chandigarh, India) for doing site-directed mutagenesis studies on VHb and with Y. Oori (Kyoto, Japan) for testing the oxygen binding kinetics of these mutants. We hope to continue these studies and obtain funding from other government agencies which are interested in sponsoring work on bioremediation.

INVENTIONS OR PATENT DISCLOSURES

None